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UTILITY PATENT APPLICATION TRANSMITTAL

Submit an original and a duplicate for fee processing

(Only for new nonprovisional applications under 37 CFR 1.53(b))

ADDRESS TO:

Assistant Commissioner for Patents
Box Patent Application
Washington, D.C. 20231

Attorney Docket No. 98,009-B1

First Named Inventor Kathleen E. Rodgers, et al.

Express Mail No. **EL118813405US**

Total Pages 62

APPLICATION ELEMENTS	ACCOMPANYING APPLICATION PARTS
1. ☒ Transmittal Form with Fee	8. ☐ Assignment Papers
2. ☒ Specification (including claims and abstract) [Total Pages 36]	9. ☒ Power of Attorney
3. ☒ Drawings [Total Sheets 6]	10. ☐ English Translation Document (if applicable)
4. ☒ Oath or Declaration [Total Pages 3]	11. ☐ Information Disclosure Statement (IDS)
a. ☐ Newly executed	☐ PTO-1449 Form
b. ☒ Copy from prior application **[Note Boxes 5 and 17 below]**	☐ Copies of IDS Citations
i. ☐ Deletion of Inventor(s) Signed statement attached deleting inventor(s) named in the prior application	12. ☒ Preliminary Amendment
5. ☒ Incorporation by Reference: The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.	13. ☒ Return Receipt Postcard (Should be specifically itemized)
6. ☐ Microfiche Computer Program	14. ☒ Small Entity Statement(s)
7. ☒ Nucleotide and/or Amino Acid Sequence Submission	☐ Enclosed
a. ☒ Computer Readable Copy	☒ Statement filed in prior application; status still proper and desired
b. ☒ Paper Copy	15. ☐ Certified Copy of Priority Document(s)
c. ☒ Statement verifying above copies	16. ☐ Other:

17. ☒ This is a CONTINUING APPLICATION. Please note the following:

a. ☒ This is a ☒ Continuation ☐ Divisional ☐ Continuation-in-part of prior application 09/245,680. This application claims priority from U.S. Provisional Patent Application Serial Nos. 60/074,106 filed February 8, 1998 and 60/111,535, filed December 9, 1998.

b. ☒ Cancel in this application original claims 3-30 of the prior application before calculating the filing fee.

c. ☒ Amend the specification by inserting before the first line the sentence: This is a ☒ continuation ☐ divisional ☐ continuation-in-part of application Serial No. 09/245,680

d. ☒ The prior application is assigned of record to **University of Southern California**

UTILITY PATENT APPLICATION TRANSMITTAL | Attorney Docket No. 98,009-B1

APPLICATION FEES

BASIC FEE				$ 690.00
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total Claims	16 - 20=	0	x $18.00	$
Independent Claims	1 - 3=	0	x $78.00	$
☐ Multiple Dependent Claims(s) if applicable			+$270.00	$
			Total of above calculations =	$ 690.00
			Reduction by 50% for filing by small entity =	$(345.00)
☐ Assignment fee if applicable			+ $40.00	$
			TOTAL =	$345.00

18. ☐ Please charge my Deposit Account No. 13-2490 in the amount of $

19. ☒ A check in the amount of $ **345.00** is enclosed.

20. The Commissioner is hereby authorized to credit overpayments or charge any additional fees of the following types to Deposit Account No. 13-2490:
    a. ☒ Fees required under 37 CFR 1.16.
    b. ☒ Fees required under 37 CFR 1.17.
    c. ☒ Fees required under 37 CFR 1.18.

21. ☒ The Commissioner is hereby generally authorized under 37 CFR 1.136(a)(3) to treat any future reply in this or any related application filed pursuant to 37 CFR 1.53 requiring an extension of time as incorporating a request therefor, and the Commissioner is hereby specifically authorized to charge Deposit Account No. 13-2490 for any fee that may be due in connection with such a request for an extension of time.

22. CERTIFICATE OF MAILING

I hereby certify that I directed that the correspondence identified above be deposited with the United States Postal Service as "Express Mail Post Office to Addressee" under 37 CFR § 1.10 on the date indicated below and is addressed to the Asst. Commissioner for Patents, Box Patent Application, Washington, DC 20231.

23. USPTO CUSTOMER NUMBER

PATENT & TRADEMARK OFFICE

020306

24. CORRESPONDENCE ADDRESS

Name	**McDonnell Boehnen Hulbert & Berghoff**
Address	300 South Wacker Drive, Suite 3200
City, State, Zip	Chicago, Illinois 60606

25. SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED

Name Reg. No.	David S. Harper 42,636
Signature	

JC924 U.S. PTO
09/08/00

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A

PATENT

JC905 U.S. PTO
09/658315
09/08/00

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
(Case No. 98,009-B1)

In re Application of:			
	Kathleen Rodgers, et al.		
		Art Unit:	To be assigned
Serial No.:	To be assigned		
		Examiner:	To be assigned
Filed:	Herewith		
For:	Method of Promoting Erythropoiesis		

Asst. Commissioner for Patents
BOX: New Application
Washington, D.C. 20231-9999

TRANSMITTAL LETTER

1. We are transmitting herewith the attached papers for the above identified new patent application:

   - ☒ Utility Patent Application Transmittal Cover
   - ☒ Specification (36 sheets)
   - ☒ Drawings (6 Figures)
   - ☒ Power of Attorney (3 sheets)
   - ☒ Preliminary Amendment (7 sheets)
   - ☒ Sequence Listing paper copy and computer readable form diskette(10 sheets)
   - ☒ Submission of Sequence Listing Statement (1 sheet)
   - ☒ Return receipt postcard

2. ☒ A check in the amount of $345.00 is enclosed.

3. **GENERAL AUTHORIZATION TO CHARGE OR CREDIT FEES:** Please charge any additional fees or credit overpayment to Deposit Account No. **13-2490**. A duplicate copy of this sheet is enclosed.

4. **CERTIFICATE OF MAILING BY "EXPRESS MAIL" UNDER 37 CFR § 1.10:** The undersigned hereby certifies that this Transmittal Letter and the papers, as described hereinabove, are being deposited with the United States Postal Service with sufficient postage as "Express Mail Post Office to Addressee" in an envelope addressed to: Asst. Commissioner for Patents, Box NEW APPLICATION, Washington, D.C. 20231, on this 8th day of September, 2000.

Express Mail No. EL118813405US

By: _____
David S. Harper
Reg. No. **42,636**

McDONNELL BOEHNEN
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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

(Case No. **98,009-B1**)

In the application of:

Kathleen E. Rodgers, et al.	)	
	)	
Serial No: **To be assigned**	)	Art Unit: **To be assigned**
	)	
Filed: **Herewith**	)	
	)	Examiner: **To be assigned**
For: **METHOD OF PROMOTING ERYTHROPOIESIS**	)	
	)	

Preliminary Amendment

Assistant Commissioner for Patents
Washington, D.C. 20231-9999

Dear Sir:

Please enter the following preliminary amendments to the specification and claims:

In the Specification:

Please delete lines 6-8 on page 1 and substitute the following: "This application is a continuation of U.S. Patent Application Serial No. 09/245,680, which claims priority from U.S. Patent Application Serial Nos. 60/074,106 filed February 8, 1998 and 60/111,535 filed December 9, 1998."

In the claims:

Please cancel claims 3-30 without prejudice to their filing in subsequent continuation or divisional applications.

Please amend the claims as follows:

1. (Amended) A method for augmenting erythropoiesis comprising contacting erythroid progenitor cells with an amount effective to augment erythropoiesis of at least one active

agent comprising a sequence [consisting] of at least three contiguous amino acids of groups R^1-R^8 in the sequence of general formula I

$$R^1\text{-}R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7\text{-}R^8$$

[in which R^1 and R^2 together form a group of formula

$$X\text{-}R^A\text{-}R^B\text{-},$$

wherein X is H or a one to three peptide group, or is absent;]

wherein R^1 is selected from the group consisting of Asp, Glu, Asn, Acpc (1-aminocyclopentane carboxylic acid), Ala, Me^2Gly, Pro, Bet, Glu(NH_2), Gly, Asp(NH_2) and Suc, or R^1 is absent;

R^2 is selected from the group consisting of Arg, Lys, Ala, Orn, Ser(Ac), Sar, D-Arg and D-Lys;

R^3 is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc and Tyr;

R^4 is selected from the group consisting of Tyr, Tyr(PO_3)$_2$, Thr, Ser, homoSer and azaTyr;

R^5 is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

R^6 is selected from the group consisting of His, Arg or 6-NH_2-Phe;

R^7 is selected from the group consisting of Pro or Ala; and

R^8 is selected from the group consisting of Phe, Phe(Br), Ile, Tyr, or is absent,

and wherein the active agent is not SEQ ID NO:1 or SEQ ID NO:19.

2. (Amended) The method of claim 1 wherein the active agent comprises a sequence [is] selected from the group consisting of angiotensinogen, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, [SEQ ID NO:19] SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO: 32, SEQ ID NO:33, SEQ ID NO: 34; SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, and SEQ ID NO:39.

Please add the following new claims:

31. The method of claim 1 wherein the active agent comprises a sequence of at least four contiguous amino acids of groups R^1-R^8 in the sequence of general formula I.

32. The method of claim 1 wherein the active agent comprises a sequence of at least five contiguous amino acids of groups R^1-R^8 in the sequence of general formula I.

33. The method of claim 1 wherein the active agent comprises a sequence of at least six contiguous amino acids of groups R^1-R^8 in the sequence of general formula I.

34. The method of claim 1 wherein the active agent comprises a sequence of at least seven contiguous amino acids of groups R^1-R^8 in the sequence of general formula I.

35. The method of claim 1 wherein the active agent consists essentially of a sequence of at least three contiguous amino acids of groups R^1-R^8 in the sequence of general formula I.

36. The method of claim 1 wherein the active agent consists essentially of a sequence of at least four contiguous amino acids of groups R^1-R^8 in the sequence of general formula I.

37. The method of claim 1 wherein the active agent consists essentially of a sequence of at least five contiguous amino acids of groups R^1-R^8 in the sequence of general formula I.

38. The method of claim 1 wherein the active agent consists essentially of a sequence of at least six contiguous amino acids of groups R^1-R^8 in the sequence of general formula I.

39. The method of claim 1 wherein the active agent consists essentially of a sequence of at least seven contiguous amino acids of groups R^1-R^8 in the sequence of general formula I.

40. The method of claim 1 wherein the contacting occurs in vivo and a dosage of active agent is between about 0.1 ng/kg and about 10.0 mg/kg.

41. The method of claim 1 wherein the contacting occurs in vitro and a dosage of active agent is between about 0.1 ng/ml and about 10.0 mg/ml.

42. The method of claim 1 further comprising contacting the erythroid progenitor cells with an amount effective to augment erythropoiesis of erythropoietin.

43. The method of claim 1, wherein the method is used to treat anemia associated with a condition selected from the group consisting of chronic renal failure, end-stage renal disease, renal transplantation, cancer, acquired immune deficiency syndrome, chemotherapy, radiotherapy, and bone marrow transplantation.

44. The method of claim 43, further comprising contacting the erythroid progenitor cells with an amount effective to augment erythropoiesis of erythropoietin.

The amendments are made simply to clarify the claims, or to add further limitations to the originally filed claims, and thus do not constitute new matter. The amendment to claim 1 to delete the term "consisting" from the phrase "comprising a sequence [consisting] of at least three contiguous amino acids..." was made to clarify that the active agent can include additional amino acids in addition to the requisite number of amino acids from the general formula. A similar amendment was made to clarify the scope of dependent claim 2. New claims 31-42 add further limitations to claim 1. The addition of claim 44 reciting contacting the cells with "erythropoietin" are supported, for example, at page 3, lines 18-22; page 15 line 19 to page 16 line 3. The addition of claim 43 reciting use of the method to treat anemia associated with the recited conditions is supported on page 3, lines 8-15 and elsewhere in the specification. Thus, the amendments and added claims do not constitute new matter.

REMARKS

In the parent case, the Examiner rejected claims 1-5 and 7 under 35 USC §103(a) as being obvious over Mrug et al. in light of Plucinska et al. Mrug teaches angiotensin II (AII), with isoleucine at position 5 to stimulate erythropoiesis. The Examiner asserted that the Plucinska reference teaches the peptide of SEQ ID NO:19, and that this peptide binds to the AT1 receptor. The Examiner further asserted that the similarity between AII and SEQ ID NO:19, coupled with their binding to the same receptor, would make it

obvious to use SEQ ID NO:19 to augment erythropoiesis. The Applicants traverse this rejection.

Neither of the cited references teaches or suggests the use of SEQ ID NO:19 to augment erythropoiesis, and combining the references does not cure the deficiency. Mrug teaches AII only to augment erythropoiesis, with no disclosure of SEQ ID NO:19. Plucinska et al. does not provide any discussion relating to erythropoiesis. Thus, the combination of references in no way makes obvious the desirability of using SEQ ID NO:19 for augmenting erythropoiesis.

However, solely to expedite allowance of certain aspects of the instant application, the claims have been amended to exclude active agents comprising the amino acid sequence of SEQ ID NO:19 for augmenting erythropoiesis. The Applicants reserve the right to pursue claims encompassing SEQ ID NO:19, in subsequent continuation applications.

3. Co-pending applications

The Applicants hereby notify the Examiner of the following commonly owned, co-pending applications that relate to the use of the active agents for other purposes:

09/210,249 filed December 11, 1998
09/198,806 filed November 24, 1998
09/012,400 filed January 23, 1998
09/264,563 filed March 8, 1999
09/287,674 filed April 7, 1999
09/307,940 filed May 10, 1999
09/246,162 filed February 8, 1999
09/255,136 filed February 19,1999
09/245,680 filed February 8, 1999
09/250,703 filed February 15, 1999
09/246,525 filed February 8, 1999
09/266,293 filed March 11, 1999

09/332,582 filed June 14, 1999
09/373,962 filed August 13, 1999
09/352,191 filed July 12, 1999
08/126,370 filed September 24, 1993
09/208,337 filed December 9, 1998
09/108,478 filed June 30, 1998
09/503,872 filed February 14, 2000
08/990,664 filed December 15, 1997

Based upon the foregoing remarks and amendments, the Applicants believe that the application is now in condition for allowance. If there is any problem, the examiner is respectfully invited to call the below signed attorney at (312) 913-2106.

Respectfully submitted,

McDonnell Boehnen Hulbert & Berghoff

Date: 9/8/2000

By: _____

David S. Harper
Registration No. 42636

PATENT

APPLICATION FOR UNITED STATES LETTERS PATENT IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

(Case No. 98,009-B)

Title: **METHOD OF PROMOTING ERYTHROPOIESIS**

Inventors:

Kathleen E. Rodgers
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Who is a citizen of the United States

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MBHB CASE NO. 98,009-B

METHOD OF PROMOTING ERYTHROPOIESIS

Cross Reference

This application is a continuation-in-part of U.S. Provisional Application Serial No. 60/074,106 filed February 9, 1998 and a continuation of U.S. Provisional Application Serial No. 60/111,535 filed December 9, 1998.

Field of the Invention

The present invention relates to compounds, methods, compositions, and kits for the stimulation of erythropoiesis. More specifically, the present invention relates to methods, compositions, and kits that employ effective amounts of angiotensinogen, angiotensin I (AI), AI analogues, AI fragments and analogues thereof, angiotensin II analogues, AII fragments or analogues thereof or AII AT_2 type 2 receptor agonists for stimulating erythropoiesis.

Background of the Invention

Maintenance of an adequate supply of oxygen to the body tissues is vital to survival. In the United States alone, several million people suffer from anemia secondary to renal failure, chronic inflammatory disease and malignancies (U.S. Patent No. 4,987,121, hereby incorporated by reference in its entirety). Since to a large degree the oxygen-carrying capacity of blood is governed by the concentration of erythrocytes in the blood, the appropriate regulation of erythropoiesis is also crucial.

The early studies of Reissmann (Reissmann, K. R., *Blood* 5:372-80 (1950)) and Erslev (Erslev, A., *Blood* 8:349-57 (1953)) clearly demonstrated the hypoxia-induced stimulation of erythropoietin secretion. When erythropoietin is secreted from the erythropoietin-producing cells in response to hypoxia, it travels through the blood to its target organ, the hematopoietic tissues. In humans, the principal hematopoietic tissue is within the liver before birth, and in the bone marrow after birth. (Id.) There, erythropoietin binds specifically to its receptor on the erythroid progenitor cells called burst forming unit-erythroid (BFU-E) and colony-forming unit-erythroid (CFU-E) and stimulates these cells to proliferate and differentiate (Spivak, J. L., *Int. J. Cell Cloning* 4:139-66 (1986)). BFU-E are the earliest erythroid progenitors and constitute 0.01%, approximately, of the nucleated bone marrow cells. CFU-E are derived from BFU-E, account for about 0.1% of marrow cells, and are much more responsive to erythropoietin than are BFU-E (Spivak, J. L., *supra*); Sawada, K., et al., *J. Clin. Invest.* 80:357-66 (1987)).

The low erythropoietin levels always present appear sufficient for a basal erythropoiesis rate. Relatively small losses of blood do not appear to stimulate increased erythropoietin production (Kickler, T. S., et al., *J. Am. Med. Assoc.* 260:65-7 (1988)). It is only after a major blood loss that there is an increased production of erythropoietin and rate of erythropoiesis.

It has been well-established that the majority of patients with renal insufficiency and anemia have serum erythropoietin levels well below what would be expected for the degree of anemia (Caro, J., et al., *J. Lab. Clin. Med.* 93:449-58 (1979); Radtke, H. W., et al., Blood 54:877-84 (1979); Chandra, M., et al., *J. Pediatr.* 113:1015-21 (1988)),

although they can still respond to hypoxia with an increase in serum erythropoietin levels (Radtke, H. W., et al., *Blood* 54:877-84 (1979); Chandra, M., et al., *J Pediatr* 113:1015-21 (1988)). However, this markedly blunted erythropoietin response substantially contributes to the pathogenesis of the anemia (Eschbach, J. W., et al., *Am J Kid Dis* 11:203-9 (1988)). As a result, patients suffering from chronic renal failure and end-stage renal disease, or those undergoing renal transplantation, develop severe anemia and require regular blood transfusions (Royet, U.S. Patent No. 5,482,924).

The use of recombinant human erythropoietin has facilitated treatment of these patients. However, recombinant erythropoietin treatment is extremely costly, and methods that augment the effect of erythropoiesis will permit the use of smaller doses of erythropoietin, and thus will decrease treatment costs. Additionally, increasing the rate of erythropoiesis would significantly improve clinical benefits for the treatment of congenital or acquired aplastic or hypoplastic anemia associated with chronic renal failure, end-stage renal disease, renal transplantation, cancer, AIDS, chemotherapy, radiotherapy, bone marrow transplantation and chronic diseases.

Summary of the Invention

In one aspect, the present invention provides compounds and methods for the augmentation of erythropoiesis by potentiating erythropoietin-induced differentiation with angiotensinogen, angiotensin I (AI), AI analogues, AI fragments and analogues thereof, angiotensin II analogues, AII fragments or analogues thereof or AII AT_2 type 2 receptor agonists as a therapeutic adjunct.

In another aspect, the present invention provides pharmaceutical compositions comprising angiotensinogen, angiotensin I (AI), AI analogues, AI fragments and analogues thereof, angiotensin II analogues, AII fragments or analogues thereof or AII AT_2 type 2 receptor agonists together with erythropoietin and a pharmaceutically acceptable carrier.

In a further aspect, the present invention provides kits for promoting erythropoiesis, wherein the kits comprise an effective amount of angiotensinogen, AI, AI analogues, AI fragments and analogues thereof, AII analogues, AII fragments or analogues thereof or AII AT_2 type 2 receptor agonists, and instructions for using the amount effective of active agent as a therapeutic adjunct to erythropoietin treatment.

In another aspect, the invention provides improved cell culture medium for promoting erythropoiesis, comprising adding an effective amount of the active agents of the invention to promote erythropoiesis.

The methods and kits of the present invention are clinically useful as a therapeutic adjunct for increasing red blood cell production in treating congenital or acquired aplastic or hypoplastic anemia.

Brief Description of the Figures

Figure 1 is a graph showing the effect of 1GD on formation of human burst-forming units-erythroid.

Figure 2 is a graph showing the effect of 24B on formation of human burst-forming units-erythroid.

Figure 3 is a graph showing the effect of 2GD on formation of human burst-forming units-erythroid.

Figure 4 is a graph showing the effect of 5GD on formation of human burst-forming units-erythroid.

Figure 5 is a graph showing the effect of AII(1-7) on formation of human burst-forming units-erythroid.

Figure 6 is a graph showing the effect of AII on formation of human burst-forming units-erythroid.

Detailed Description of the Preferred Embodiments

All cited patents, patent applications and references are hereby incorporated by reference in their entirety.

As defined herein, the term "erythropoiesis" refers to red blood cell production.

As defined herein, "augmentation of erythropoiesis" may occur either by direct stimulation of erythroid production, by increasing erythropoietin production, or by any other mechanism.

Unless otherwise indicated, the term "active agents" as used herein refers to the group of compounds comprising angiotensinogen, angiotensin I (AI), AI analogues, AI fragments and analogues thereof, angiotensin II analogues, AII fragments or analogues thereof and AII AT_2 type 2 receptor agonists.

The biological formation of angiotensin is initiated by the action of renin on the plasma substrate angiotensinogen (*Circulation Research* 60:786-790 (1987); Clouston et al., Genomics 2:240-248 (1988); Kageyama et al., *Biochemistry* 23:3603-3609; Ohkubo

et al., *Proc. Natl. Acad. Sci.* 80:2196-2200 (1983); all references hereby incorporated in their entirety). The substance so formed is a decapeptide called angiotensin I (AI) which is converted to AII by the converting enzyme angiotensinase which removes the C-terminal His-Leu residues from AI (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu [SEQ ID NO:37]). AII is a known pressor agent and is commercially available.

Angiotensin-converting enzyme (ACE) inhibitors have been observed to exacerbate anemia in patients with chronic renal failure and end-stage renal disease (ESRD), as well as in renal transplant recipients (Cruz et al., *Am. J. Kidney Diseases* 28:535-540 (1996); herein incorporated by reference in its entirety). ACE inhibitors appear to induce anemia by decreasing red blood cell production (*Id.*) Some data exist which suggest that ACE inhibitors may reduce red blood cell production by inhibiting angiotensin-mediated erythropoietin synthesis. (Hirikata et al., *Clin. Nephrol.* 26:27-32 (1986); Gould et al., *J. Lab. Clin. Med.* 96:523-534 (1980); Conlon et al., *Transplantation* 56:217-219 (1993)). However, other studies show that ACE inhibitors do not inhibit erythropoietin synthesis, and suggest that angiotensin does not play a role in erythropoiesis. (Cruz et al., 1996; Anderson et al., *Biology of the Neonate* 71:194-197 (1997); Shand et al., *J. Hum. Hypertension* 9:233-235 (1995); Julian et al., *Kidney Int.* 46:1397-1403 (1994); Gaston et al., *Transplant Proc.* 25:1029-1031 (1993); Islam et al., *Transplant Int.* 3:222-225 (1990); Rostaing et al., *Transplant Proc.* 26:280-281 (1994)). Thus, it is unclear whether AII, or any AII analogues and fragments, stimulate production of erythropoietin.

A recent study suggests that activation of the AT1 receptor with angiotensin II enhances erythropoietin-stimulated human erythroid proliferation *in vitro*. (Mrug et al.,

J. Clin. Invest. 100 (9):2310-2314 (1997). Previous studies have indicated that slow infusion of angiotensin II in dogs (Fisher et al., in Annals New York Academy of Sciences, pp. 308-317: 1968) and mice (Fisher et al., *J. Pharmacol. and Exper. Therapeutics* 157:618-625, 1967) led to increased erythropoietin production, while injections of angiotensin II into mice and rats did not lead to such an increase. (Mann et al., P.S.E.B.M. 121:1152-1154 (1966); Bilsel et al., P.S.E.B.M. 114:475-479 (1963)). Thus, there is some evidence that angiotensin II (AII) stimulates erythropoiesis *in vitro*, while the *in vivo* data is unclear. However, there is no data suggesting whether any AII analogues and fragments also stimulate erythropoiesis, whether *in vitro* or *in vivo*. For example, data suggests that the AII fragment AII(1-7) acts through a receptor(s) that is distinct from the AT1 and AT2 receptors which modulate AII activity. (Ferrario et al., J. Am. Soc. Nephrol. 9:1716-1722 (1998); Iyer et al., Hypertension 31:699-705 (1998); Freeman et al., Hypertension 28:104 (1996); Ambuhl et al., Brain Res. Bull. 35:289 (1994). Thus, the stimulatory effect of AII through activation of the AT1 receptor reported by Mrug et al. (see above) does not shed any light on the potential stimulatory effect of AII(1-7).

U.S. Patent No. 5,015,629 to DiZerega (the entire disclosure of which is hereby incorporated by reference) describes a method for increasing the rate of healing of wound tissue, comprising the application to such tissue of angiotensin II (AII) in an amount which is sufficient for said increase. The application of AII to wound tissue significantly increases the rate of wound healing, leading to a more rapid re-epithelialization and tissue repair. The term AII refers to an octapeptide present in humans and other species having the sequence Asp-Arg-Val-Tyr-Ile-His-Pro-Phe [SEQ ID NO:1]. The use of AII

analogues and fragments, AT2 agonists, as well as AIII and AIII analogues and fragments in wound healing has also been described. (U.S. Patent No. 5,629,292; U.S. Patent No. 5,716,935; WO 96/39164; all references herein incorporated by reference in their entirety.)

A peptide agonist selective for the AT2 receptor (AII has 100 times higher affinity for AT2 than AT1) has been identified. This peptide is p-aminophenylalanine6-AII ["(p-NH_2-Phe)6-AII"], Asp-Arg-Val-Tyr-Ile-Xaa-Pro-Phe [SEQ ID NO.36] wherein Xaa is p-NH_2-Phe (Speth and Kim, BBRC 169:997-1006 (1990)). This peptide gave binding characteristics comparable to AT2 antagonists in the experimental models tested (Catalioto, et al., *Eur. J. Pharmacol.* 256:93-97 (1994); Bryson, et al., *Eur. J. Pharmacol.* 225:119-127 (1992)).

Many studies have focused upon AII(1-7) (AII residues 1-7) or other fragments of AII to evaluate their activity. AII(1-7) elicits some, but not the full range of effects elicited by AII. Pfeilschifter, et al., *Eur. J. Pharmacol.* 225:57-62 (1992); Jaiswal, et al., *Hypertension* 19 (Supp. II):II-49-II-55 (1992); Edwards and Stack, *J. Pharmacol. Exper. Ther.* 266:506-510 (1993); Jaiswal, et al., *J. Pharmacol. Exper. Ther.* 265:664-673 (1991); Jaiswal, et al., *Hypertension* 17:1115-1120 (1991); Portsi, et al., *Br. J. Pharmacol.* 111:652-654 (1994).

The active AII analogues, fragments of AII and analogues thereof of particular interest in accordance with the present invention are characterized as comprising a sequence consisting of at least three contiguous amino acids of groups R^1-R^8 in the sequence of general formula I

$$R^1\text{-}R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7R^8$$

in which R^1 and R^2 together form a group of formula

$$X\text{-}R^A\text{-}R^B\text{-},$$

wherein X is H or a one to three peptide group,

R^A is suitably selected from Asp, Glu, Asn, Acpc (1-aminocyclopentane carboxylic acid), Ala, Me^2Gly, Pro, Bet, Glu(NH_2), Gly, Asp(NH_2) and Suc,

R^B is suitably selected from Arg, Lys, Ala, Orn, Ser(Ac), Sar, D-Arg and D-Lys;

R^3 is selected from the group consisting of Val, Ala, Leu, Lys, norLeu, Ile, Gly, Pro, Aib, Acpc and Tyr;

R^4 is selected from the group consisting of Tyr, Tyr(PO_3)$_2$, Thr, Ser, Ala, homoSer and azaTyr;

R^5 is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

R^6 is His, Arg or 6-NH_2-Phe;

R^7 is Pro or Ala; and

R^8 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, excluding sequences including R^4 as a terminal Tyr group.

Compounds falling within the category of AT2 agonists useful in the practice of the invention include the AII analogues set forth above subject to the restriction that R^6 is p-NH_2-Phe.

Particularly preferred combinations for R^A and R^B are Asp-Arg, Asp-Lys, Glu-Arg and Glu-Lys. Particularly preferred embodiments of this class include the following: AIII, Arg-Val-Tyr-Ile-His-Pro-Phe [SEQ ID NO:2]; AII(3-8), also known as des1-AIII

or AIV, Val-Tyr-Ile-His-Pro-Phe [SEQ ID NO:3]; AII(1-7), Asp-Arg-Val-Tyr-Ile-His-Pro {SEQ ID NO:4]; AII(2-7). Arg-Val-Tyr-Ile-His-Pro [SEQ ID NO:5]; AII(3-7), Val-Tyr-Ile-His-Pro [SEQ ID NO:6]; AII(5-8), Ile-His-Pro-Phe [SEQ ID NO:7]; AII(1-6), Asp-Arg-Val-Tyr-Ile-His [SEQ ID NO:8]; AII(1-5), Asp-Arg-Val-Tyr-Ile [SEQ ID NO:9]; AII(1-4), Asp-Arg-Val-Tyr [SEQ ID NO:10]; and AII(1-3), Asp-Arg-Val [SEQ ID NO:11]. Other preferred embodiments include: Arg-norLeu-Tyr-Ile-His-Pro-Phe [SEQ ID NO:12] and Arg-Val-Tyr-norLeu-His-Pro-Phe [SEQ ID NO:13]. Still another preferred embodiment encompassed within the scope of the invention is a peptide having the sequence Asp-Arg-Pro-Tyr-Ile-His-Pro-Phe [SEQ ID NO:31]. AII(6-8), His-Pro-Phe [SEQ ID NO:14] and AII(4-8), Tyr-Ile-His-Pro-Phe [SEQ ID NO:15] were also tested and found not to be effective.

A further class of particularly preferred compounds in accordance with the present invention consists of those with the following general structure:

ASP-ARG-R1-R2-R3-R4-PRO-R5

wherein R1 is selected from the group consisting of Val, Pro, and Lys;

R2 is selected from the group consisting of Tyr, Tyr $(PO_3)_2$ and Ala;

R3 is selected from the group consisting of Ile, Val, Leu, norLeu and Ala;

R4 is selected from the group consisting of His and Arg; and

R5 is either Phe or is absent,

and wherein the active agent is not AII.

Preferred embodiments of this class of the invention include SEQ ID NO:4, SEQ ID NO:19, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:31, SEQ ID NO: 32, SEQ ID NO: 34; SEQ ID NO:38, SEQ ID NO:39, and SEQ ID NO. 40. Particularly preferred

embodiments of this class include SEQ ID NO:4, SEQ ID NO. 31, SEQ ID NO 38, and SEQ ID NO. 39.

Another class of compounds of particular interest in accordance with the present invention are those of the general formula II

$$R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7\text{-}R^8$$

in which R^2 is selected from the group consisting of H, Arg, Lys, Ala, Orn, Ser(Ac), Sar, D-Arg and D-Lys;

R^3 is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc and Tyr;

R^4 is selected from the group consisting of Tyr, $Tyr(PO_3)_2$, Thr, Ser, homoSer and azaTyr;

R^5 is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

R^6 is His, Arg or 6-NH_2-Phe;

R^7 is Pro or Ala; and

R^8 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr.

A particularly preferred subclass of the compounds of general formula II has the formula

$$R^2\text{-}R^3\text{-Tyr-}R^5\text{-His-Pro-Phe}$$ [SEQ ID NO:16]

wherein R^2, R^3 and R^5 are as previously defined. Particularly preferred is angiotensin III of the formula Arg-Val-Tyr-Ile-His-Pro-Phe [SEQ ID NO:2]. Other

preferred compounds include peptides having the structures Arg-Val-Tyr-Gly-His-Pro-Phe [SEQ ID NO:17] and Arg-Val-Tyr-Ala-His-Pro-Phe [SEQ ID NO:18]. The fragment AII(4-8) was ineffective in repeated tests; this is believed to be due to the exposed tyrosine on the N-terminus.

In the above formulas, the standard three-letter abbreviations for amino acid residues are employed. In the absence of an indication to the contrary, the L-form of the amino acid is intended. Other residues are abbreviated as follows:

TABLE 1

Abbreviation for Amino Acids

Me^2Gly	N,N-dimethylglycyl
Bet	1-carboxy-N,N,N-trimethylmethanaminium hydroxide inner salt (betaine)
Suc	Succinyl
Phe(Br)	p-bromo-L-phenylalanyl
azaTyr	aza-α'-homo-L-tyrosyl
Acpc	1-aminocyclopentane carboxylic acid
Aib	2-aminoisobutyric acid
Sar	N-methylglycyl (sarcosine)

It has been suggested that AII and its analogues adopt either a *gamma* or a *beta* turn (Regoli, et al., Pharmacological Reviews 26:69 (1974). In general, it is believed that neutral side chains in position R^3, R^5 and R^7 may be involved in maintaining the appropriate distance between active groups in positions R^4, R^6 and R^8 primarily responsible for binding to receptors and/or intrinsic activity. Hydrophobic side chains in

positions R^3, R^5 and R^8 may also play an important role in the whole conformation of the peptide and/or contribute to the formation of a hypothetical hydrophobic pocket.

Appropriate side chains on the amino acid in position R^2 may contribute to affinity of the compounds for target receptors and/or play an important role in the conformation of the peptide. For this reason, Arg and Lys are particularly preferred as R^2.

For purposes of the present invention, it is believed that R^3 may be involved in the formation of linear or nonlinear hydrogen bonds with R^5 (in the gamma turn model) or R^6 (in the beta turn model). R^3 would also participate in the first turn in a beta antiparallel structure (which has also been proposed as a possible structure). In contrast to other positions in general formula I, it appears that beta and gamma branching are equally effective in this position. Moreover, a single hydrogen bond may be sufficient to maintain a relatively stable conformation. Accordingly, R^3 may suitably be selected from Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc and Tyr. Furthermore, Lys has surprisingly been found to be suitable at R^3 (see Examples).

With respect to R^4, conformational analyses have suggested that the side chain in this position (as well as in R^3 and R^5) contribute to a hydrophobic cluster believed to be essential for occupation and stimulation of receptors. Thus, R^4 is preferably selected from Tyr, Thr, Tyr $(PO_3)_2$, homoSer, Ser and azaTyr. Furthermore, Ala has surprisingly been found to be suitable at the R^4 position (See Examples). In this position, Tyr is particularly preferred as it may form a hydrogen bond with the receptor site capable of accepting a hydrogen from the phenolic hydroxyl (Regoli, et al. (1974), *supra*).

In position R^5, an amino acid with a β aliphatic or alicyclic chain is particularly desirable. Therefore, while Gly is suitable in position R^5, it is preferred that the amino acid in this position be selected from Ile, Ala, Leu, norLeu, Gly and Val.

In the AI and AII analogues, fragments and analogues of fragments of particular interest in accordance with the present invention, R^6 is His, Arg or 6-NH_2-Phe. The unique properties of the imidazole ring of histidine (e.g., ionization at physiological pH, ability to act as proton donor or acceptor, aromatic character) are believed to contribute to its particular utility as R^6. For example, conformational models suggest that His may participate in hydrogen bond formation (in the *beta* model) or in the second turn of the antiparallel structure by influencing the orientation of R^7. Similarly, it is presently considered that R^7 should be Pro in order to provide the most desirable orientation of R^8. In position R^8, both a hydrophobic ring and an anionic carboxyl terminal appear to be particularly useful in binding of the analogues of interest to receptors; therefore, Tyr and especially Phe are preferred for purposes of the present invention.

Analogues of particular interest include the following:

TABLE 2

Angiotensin II Analogues

AII Analogue Name	Amino Acid Sequence	Sequence Identifier
Analogue 1	Asp-Arg-Val-Tyr-Val-His-Pro-Phe	SEQ ID NO: 19
Analogue 2	Asn-Arg-Val-Tyr-Val-His-Pro-Phe	SEQ ID NO: 20
Analogue 3	Ala-Pro-Gly-Asp-Arg-Ile-Tyr-Val-His-Pro-Phe	SEQ ID NO: 21
Analogue 4	Glu-Arg-Val-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 22
Analogue 5	Asp-Lys-Val-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 23
Analogue 6	Asp-Arg-Ala-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 24
Analogue 7	Asp-Arg-Val-Thr-Ile-His-Pro-Phe	SEQ ID NO: 25
Analogue 8	Asp-Arg-Val-Tyr-Leu-His-Pro-Phe	SEQ ID NO: 26
Analogue 9	Asp-Arg-Val-Tyr-Ile-Arg-Pro-Phe	SEQ ID NO: 27

Analogue 10	Asp-Arg-Val-Tyr-Ile-His-Ala-Phe	SEQ ID NO: 28
Analogue 11	Asp-Arg-Val-Tyr-Ile-His-Pro-Tyr	SEQ ID NO: 29
Analogue 12	Pro-Arg-Val-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 30
Analogue 13	Asp-Arg-Pro-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 31
Analogue 14	Asp-Arg-Val-Tyr(Po_3)$_2$-Ile-His-Pro-Phe	SEQ ID NO: 32
Analogue 15	Asp-Arg-norLeu-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 33
Analogue 16	Asp-Arg-Val-Tyr-norLeu-His-Pro-Phe	SEQ ID NO: 34
Analogue 17	Asp-Arg-Val-homoSer-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 35

The polypeptides of the instant invention may be synthesized by a wide variety of methods such as those set forth in J. M. Stewart and J. D. Young, Solid Phase Peptide Synthesis, 2nd ed., Pierce Chemical Co., Rockford, Ill. (1984) and J. Meienhofer, Hormonal Proteins and Peptides, Vol. 2, Academic Press, New York, (1973) for solid phase synthesis and E. Schroder and K. Lubke, The Peptides, Vol. 1, Academic Press, New York, (1965) for solution synthesis. The disclosures of the foregoing treatises are incorporated by reference herein.

In general, these methods involve the sequential addition of protected amino acids to a growing peptide chain (U.S. Patent No. 5,693,616, herein incorporated by reference in its entirety). Normally, either the amino or carboxyl group of the first amino acid and any reactive side chain group are protected. This protected amino acid is then either attached to an inert solid support, or utilized in solution, and the next amino acid in the sequence, also suitably protected, is added under conditions amenable to formation of the amide linkage. After all the desired amino acids have been linked in the proper sequence, protecting groups and any solid support are removed to afford the crude polypeptide. The polypeptide is desalted and purified, preferably chromatographically, to yield the final product.

In one aspect, the present invention provides methods for the augmentation of erythropoiesis by potentiating erythropoietin-induced differentiation with

angiotensinogen, angiotensin I (AI), AI analogues, AI fragments and analogues thereof, angiotensin II analogues, AII fragments or analogues thereof or AII AT_2 type 2 receptor agonists (ie: "active agents") as a therapeutic adjunct to erythropoietin treatment. The methods and kits of the present invention are clinically useful as a therapeutic adjunct for increasing red blood cell production in treating congenital or acquired aplastic or hypoplastic anemia.

The use of erythropoietin to promote erythropoiesis is well known in the art, as exemplified by Royet et al., U.S. Patent No. 5,482,924; Goldberg et al., U.S. Patent No. 5,188,828; Vance et al., U.S. Patent No. 5,541,158; and Baertschi et al., U.S. Patent No. 4,987,121, all references hereby incorporated in their entirety. The erythropoietin dosage regimen may vary widely, but can be determined routinely by a physician using standard methods. Dosage levels of the order of between about 1 EPO unit/kg and about 5,000 EPO units/kg body weight are useful for all methods of use disclosed herein.

In one embodiment, the effects of the active agents on the growth of erythroid progenitors *in vitro* are tested using the colony formation assay. The assay consists of growing erythroid progenitor cells in a semi-solid medium (methylcellulose) for two weeks (Yu et al., U.S. Patent No. 5,032,507). Conditioned medium consisting of phytohemagglutinin-treated lymphocytes (PHA-LCM) is supplemented with erythropoietin and preferably, between about 0.1 ng/ml and about 10 mg/ml of the active agents.

The growth of erythroid precursors termed BFU-E (burst forming units erythroid) is monitored by identification and counting of the colonies under an inverted microscope as well as by staining of colonies (Yu et al., U.S. Patent No. 5,032,507). The number of

mixed colonies represents the number of earlier progenitor cells (containing erythroid as well as one or more other lineage cells).

In another embodiment, erythropoiesis is augmented *ex vivo* by obtaining a sample of bone marrow cells, as is known in the art, potentiating erythropoietin-induced differentiation with the active agents of the invention and infusing the treated cells back into the patient.

In a preferred embodiment, bone marrow cells are isolated from peripheral blood samples via standard techniques (U.S. Patent Nos. 4,987,121, 5,104,653; hereby incorporated by reference in their entirety). 2 x 10^6 bone marrow cells are seeded in culture dishes in appropriate medium, such as modified Dulbecco's medium (IMDM) supplemented with (final concentration): horse serum (15%), fetal calf serum (5%), Fe saturated transferrin (0.4 mg/ml) hydrocortisone, penicillin 100 u/ml, and streptomycin (0.1 mg/ml) (Royet et al., U.S. Patent No. 5,482,924). An adherent cell monolayer is formed. After 15 days, the non adherent cells are removed and fresh bone marrow is re-seeded in the presence of 0.1 U/ml of erythropoietin (EPO) and, preferably, between about 0.1 ng/ml and about 10 mg/ml of the active agents of the invention. The cells are expanded for a period of between 2 and 21 days with subsequent medium changes as required. Prior to reinfusion into the subject the cells are examined microscopically to verify the absence of contamination. The cells are rinsed to remove all traces of culture fluid, resuspended in an appropriate medium and then pelleted and rinsed several times. After the final rinse, the cells are resuspended at between 0.7 x 10^6 and 50 x 10^6 cells per ml in an appropriate medium and reinfused into a subject. Erythropoiesis is monitored by

red cell count or hemoglobin concentration with time (Yu et al., U.S. Patent No. 5,032,507, herein incorporated by reference in its entirety).

For use in increasing erythropoiesis *in vivo*, the active agents may be administered by any suitable route, including orally, parentally, by inhalation spray, rectally, transdermally, or topically in dosage unit formulations containing conventional pharmaceutically acceptable carriers, adjuvants, and vehicles. The term parenteral as used herein includes, subcutaneous, intravenous, intra-arterial, intramuscular, intrasternal, intratendinous, intraspinal, intracranial, intrathoracic, infusion techniques or intraperitoneally.

Transdermal means including, but not limited to, transdermal patches may be utilized to deliver the active agents to the treatment site. Transdermal formulations may be prepared by incorporating the active agent in a thixotropic or gelatinous carrier including, but not limited to a cellulose medium, e.g., methyl cellulose or hydroxyethyl cellulose, with the resulting formulation then being packed in a transdermal device adapted to be secured in dermal contact with the skin of a wearer.

The active agents may be made up in a solid form (including granules, powders or suppositories) or in a liquid form (*e.g.*, solutions, suspensions, or emulsions). The active agents may be subjected to conventional pharmaceutical operations such as sterilization and/or may contain conventional adjuvants, such as preservatives, stabilizers, wetting agents, emulsifiers, buffers etc.

While the active agents can be administered as the sole active pharmaceutical agent, they can also be used in combination with one or more other compounds. When administered as a combination, the active agent(s) and compound(s) can be formulated as

separate compositions that are given at the same time or different times, or the active agent(s) and compound(s) can be given as a single composition.

For administration, the active agents are ordinarily combined with one or more adjuvants appropriate for the indicated route of administration. The active agents may be admixed with lactose, sucrose, starch powder, cellulose esters of alkanoic acids, stearic acid, talc, magnesium stearate, magnesium oxide, sodium and calcium salts of phosphoric and sulphuric acids, acacia, gelatin, sodium alginate, polyvinylpyrrolidine, and/or polyvinyl alcohol, and tableted or encapsulated for conventional administration. Alternatively, the compounds of this invention may be dissolved in saline, water, polyethylene glycol, propylene glycol, carboxymethyl cellulose colloidal solutions, ethanol, corn oil, peanut oil, cottonseed oil, sesame oil, tragacanth gum, and/or various buffers. Other adjuvants and modes of administration are well known in the pharmaceutical art. The carrier or diluent may include time delay material, such as glyceryl monostearate or glyceryl distearate alone or with a wax, or other materials well known in the art.

Formulations suitable for topical administration include liquid or semi-liquid preparations suitable for penetration through the skin (*e.g.*, liniments, lotions, ointments, creams, or pastes) and drops suitable for administration to the eye, ear, or nose.

The dosage regimen for augmenting erythropoiesis with the active agents is based on a variety of factors, including the type of injury, the age, weight, sex, medical condition of the individual, the severity of the condition, the route of administration, and the particular compound employed. Thus, the dosage regimen may vary widely, but can be determined routinely by a physician using standard methods. Dosage levels of the

order of between 0.1 ng/kg and 10 mg/kg of the active agents per body weight are useful for all methods of use disclosed herein.

The treatment regime will vary depending on the disease being treated, based on a variety of factors, including the type of injury, the age, weight, sex, medical condition of the individual, the severity of the condition, the route of administration, and the particular compound employed.

In a preferred embodiment of the present invention, the active agents are administered intravenously. A suitable dose of the active agents is preferably between about 0.1 ng/kg and about 10 mg/kg administered twice daily. For topical administration, the active ingredient may comprise from 0.001% to 10% w/w, *e.g.*, from 1% to 2% by weight of the formulation, although it may comprise as much as 10% w/w, but preferably not more than 5% w/w, and more preferably from 0.1% to 1% of the formulation.

In another aspect, the present invention provides a novel peptide with erythropoiesis-promoting activity, consisting of the peptide with the sequence Asp-Arg-Lys-Tyr-Ile-His-Pro-Phe (SEQ ID NO:39).

A further object of the present invention is to provide pharmaceutical compositions comprising the active agents as an ingredient for use in the method of the invention. The compositions comprise the active agents together with erythropoietin and a pharmaceutically acceptable carrier, this term including any carrier which does not interfere with the effectiveness of the biological activity of the active agents and erythropoietin, and which is not toxic to the host to which it is administered. Dosage and administration of the pharmaceutical compositions will vary depending on the disease being treated, based on a variety of factors, including the type of injury, the age, weight,

sex, medical condition of the individual, the severity of the condition, the route of administration, and the particular compound employed, as above. Thus, the dosage regimen may vary widely, but can be determined routinely by a physician using standard methods.

In a further aspect, the present invention provides kits promoting erythropoiesis, wherein the kits comprise an effective amount of the active agent, and instructions for using the amount effective of active agent as a therapeutic adjunct. In a preferred embodiment, the kit further comprises a pharmaceutically acceptable carrier, such as those adjuvants described above. In another preferred embodiment, the kit further comprises a means for delivery of the active agent to a mammal. Such devices include, but are not limited to matrical or micellar solutions, polyethylene glycol polymers, carboxymethyl cellulose preparations, crystalloid preparations (e.g., saline, Ringer's lactate solution, phosphate-buffered saline, etc.), viscoelastics, polyethylene glycols, and polypropylene glycols.

In a further preferred embodiment, the kits also comprise an amount of erythropoietin effective to accelerate erythropoiesis.

In another aspect of the present invention, an improved cell culture medium is provided for the promotion of erythropoiesis, wherein the improvement comprises addition to the cell culture medium of an effective amount of the active agents of the invention. Any cell culture media that can support erythropoiesis can be used with the present invention. Such cell culture media include, but are not limited to Basal Media Eagle, Dulbecco's Modified Eagle Medium, Iscove's Modified Dulbecco's Medium,

McCoy's Medium, Minimum Essential Medium, F-10 Nutrient Mixtures, Opti-MEM® Reduced-Serum Medium, and RPMI Medium, or combinations thereof.

The improved cell culture medium can be supplied in either a concentrated (ie: 10X) or non-concentrated form, and may be supplied as a liquid, a powder, or a lyophilizate. The cell culture may be either chemically defined, or may contain a serum supplement. Culture media and serum supplements are commercially available from many sources, such as GIBCO BRL (Gaithersburg, MD) and Sigma (St. Louis, MO).

The present invention, by providing methods and pharmaceutical compositions for augmenting erythropoiesis, will greatly increase the clinical benefits of treatment for congenital or acquired aplastic or hypoplastic anemia; amelioration of anemia associated with cancer, AIDS, chemotherapy, radiotherapy, bone marrow transplantation and chronic diseases.

The present invention may be better understood with reference to the accompanying examples that are intended for purposes of illustration only and should not be construed to limit the scope of the invention, as defined by the claims appended hereto.

Example 1. AII and AII analogue and fragment effect on erythroid progenitor formation

CD34+ cells were isolated from human cord blood by immunomagnetic chromatography using an antibody cocktail (Stem Cell Technologies, Vancouver BC) consisting of the following cell surface proteins: glycophorin A, CD56, CD66b, CD3, CD24, CD14, CD2, CD19, and CD16. Enriched cells were cultured at 37° at 5% CO_2 and air for 6 days in medium containing serum-free StemSpan (Stem Cell Technologies),

3 IU/ml human erythropoietin (EPO), 20 ng/ml stem cell factor, 20 ng/ml interleukin 3, and 20 ng/ml GM-CSF. The cells were harvested, counted and cultured in 96 well plates at a density of 50,000 cells per well in the same medium containing from 0 to 10 μg/ml of AII, AII analogues, or AII fragments. The peptides tested, and the figure showing the data received in those tests, are listed in Table 3. After 3 additional days in suspension culture (day 9), the cells were washed to remove the peptides and cultured to assess colony formation. The culture medium contained 0.9% methylcellulose in Iscove's MDM with 30% fetal calf serum, 1% bovine serum albumin, 10 μM 2-mercaptoethanol, 2mM L-glutamine, 10% Agar Leukocyte Conditioned Medium with 3 IU/ml EPO. On days 2, 4, 9, and 14 the number and size of colonies was assessed as well as the number of BFU-E formed (burst forming units-erythroid) as a measure of erythropoiesis.

Table 3. Designation for Peptides Tested

Name	Abbreviation	Sequence	Figure	SEQ ID
1GD	Ala^4-AII(1-7)	DRVAIHP	1	SEQ ID NO. 18
GSD 24B	Pro^3-AII	DRPYIHPF	2	SEQ ID NO. 31
2GD	Pro^3-AII(1-7)	DRPYIHP	3	SEQ ID NO. 38
5GD	Lys^3-AII(1-7)	DRKYIHP	4	SEQ ID NO 39
AII(1-7)		DRVYIHP	5	SEQ ID NO. 4
AII		DRVYIHPF	6	SEQ ID NO. 1

The data from these experiments are presented in Figures 1-6 and show that each of the peptides tested increased the number of BFU-E formed relative to control where no peptide was added. Each of the peptides also increased the size of the colonies assessed (data not shown). Therefore, these data demonstrate that each of the peptides tested can be used to accelerate erythroid progenitor formation, and thus to promote erythropoiesis.

The methods and kits of the present invention are clinically useful as a therapeutic adjunct for increasing red blood cell production in treating congenital or acquired aplastic or hypoplastic anemia.

The present invention is not limited by the aforementioned particular preferred embodiments. It will occur to those ordinarily skilled in the art that various modifications may be made to the disclosed preferred embodiments without diverting from the concept of the invention. All such modifications are intended to be within the scope of the present invention.

We claim:

1. A method for augmenting erythropoiesis comprising contacting erythroid progenitor cells with an amount effective to augment erythropoiesis of at least one active agent comprising a sequence consisting of at least three contiguous amino acids of groups R^1-R^8 in the sequence of general formula I

$$R^1\text{-}R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7\text{-}R^8$$

in which R^1 and R^2 together form a group of formula

$$X\text{-}R^A\text{-}R^B\text{-},$$

wherein X is H or a one to three peptide group

R^A is selected from Asp, Glu, Asn, Acpc, Ala, Me^2Gly, Pro, Bet, $Glu(NH_2)$, Gly, $Asp(NH_2)$ and Suc;

R^B is selected from Arg, Lys, Ala, Orn, Ser(Ac), Sar, D-Arg and D-Lys;

R^3 is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc, Lys and Tyr;

R^4 is selected from the group consisting of Tyr, $Tyr(PO_3)_2$, Thr, Ser, Ala, homoSer and azaTyr;

R^5 is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

R^6 is His, Arg or 6-NH_2-Phe;

R^7 is Pro or Ala; and

R^8 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, excluding sequences including R^4 as a terminal Tyr group,

and wherein the active agent is not AII.

2. The method of claim 1 wherein the active agent is selected from the group consisting of angiotensinogen, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO: 32, SEQ ID NO:33, SEQ ID NO: 34; SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, and SEQ ID NO:39.

3. The method of claim 1 wherein the concentration of active agent is between about 0.1 ng/kg and about 10.0 mg/kg.

4. A method for augmenting erythropoiesis comprising contacting erythroid progenitor cells with an amount effective to augment erythropoiesis of an active agent comprising a sequence of the following general formula:

ASP-ARG-R1-R2-R3-R4-PRO-R5

wherein R1 is selected from the group consisting of Val, Pro, and Lys;

R2 is selected from the group consisting of Tyr, Tyr $(PO_3)_2$ and Ala;

R3 is selected from the group consisting of Ile, Val, Leu, norLeu and Ala;

R4 is selected from the group consisting of His and Arg; and

R5 is either Phe or is absent,

and wherein the active agent is not AII.

5. The method of claim 4 wherein the active agent is selected from the group consisting of SEQ ID NO:4, SEQ ID NO:19, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:31, SEQ ID NO: 32, SEQ ID NO: 34; SEQ ID NO:38, and SEQ ID NO:39.

6. The method of claim 4 wherein the active agent is selected from the group consisting of SEQ ID NO:4, SEQ ID NO. 31, SEQ ID NO 38, and SEQ ID NO. 39.

7. The method of claim 4 wherein the concentration of active agent is between about 0.1 ng/kg and about 10.0 mg/kg.

8. A pharmaceutical composition comprising an amount effective to augment erythropoiesis of at least one active agent comprising a sequence consisting of at least three contiguous amino acids of groups R^1-R^8 in the sequence of general formula I

$$R^1\text{-}R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7\text{-}R^8$$

in which R^1 and R^2 together form a group of formula

$$X\text{-}R^A\text{-}R^B\text{-},$$

wherein X is H or a one to three peptide group

R^A is selected from Asp, Glu, Asn, Acpc, Ala, Me^2Gly, Pro, Bet, $Glu(NH_2)$, Gly, $Asp(NH_2)$ and Suc;

R^B is selected from Arg, Lys, Ala, Orn, Ser(Ac), Sar, D-Arg and D-Lys;

R^3 is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc, Lys and Tyr;

R^4 is selected from the group consisting of Tyr, $Tyr(PO_3)_2$, Thr, Ser, Ala, homoSer and azaTyr;

R^5 is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

R^6 is His, Arg or 6-NH_2-Phe;

R^7 is Pro or Ala; and

R^8 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, excluding sequences including R^4 as a terminal Tyr group,

wherein the active agent is not AII;

an amount of erythropoietin effective to stimulate erythropoiesis; and

a pharmaceutically acceptable carrier.

9. The pharmaceutical composition of claim 8 wherein the active agent is selected from the group consisting of angiotensinogen, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO: 32, SEQ ID NO:33, SEQ ID NO: 34; SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, and SEQ ID NO:39.

10. The pharmaceutical composition of claim 8 wherein the concentration of active agent is between about 0.1 ng/kg and about 10.0 mg/kg.

11. A pharmaceutical composition comprising an amount effective to augment erythropoiesis of an active agent comprising a sequence of the following general formula:

ASP-ARG-R1-R2-R3-R4-PRO-R5

wherein R1 is selected from the group consisting of Val, Pro, and Lys;

R2 is selected from the group consisting of Tyr, Tyr $(PO_3)_2$ and Ala;

R3 is selected from the group consisting of Ile, Val, Leu, norLeu and Ala;

R4 is selected from the group consisting of His and Arg; and

R5 is either Phe or is absent,

and wherein the active agent is not AII.

an amount of erythropoietin effective to stimulate erythropoiesis; and

a pharmaceutically acceptable carrier.

12. The pharmaceutical composition of claim 11 wherein the active agent is selected from the group consisting of SEQ ID NO:4, SEQ ID NO:19, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:31, SEQ ID NO: 32, SEQ ID NO: 34; SEQ ID NO:38, and SEQ ID NO:39.

13. The pharmaceutical composition of claim 11 wherein the active agent is selected from the group consisting of SEQ ID NO:4, SEQ ID NO. 31, SEQ ID NO 38, and SEQ ID NO. 39.

14. The pharmaceutical composition of claim 11 wherein the concentration of active agent is between about 0.1 ng/kg and about 10.0 mg/kg.

15. A kit for augmenting erythropoiesis, comprising:

(a) an amount effective to augment erythropoiesis of at least one active agent comprising a sequence consisting of at least three contiguous amino acids of groups R^1-R^8 in the sequence of general formula I

$$R^1\text{-}R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7\text{-}R^8$$

in which R^1 and R^2 together form a group of formula

$$X\text{-}R^A\text{-}R^B\text{-},$$

wherein X is H or a one to three peptide group

R^A is selected from Asp, Glu, Asn, Acpc, Ala, Me^2Gly, Pro, Bet, $Glu(NH_2)$, Gly, $Asp(NH_2)$ and Suc;

R^B is selected from Arg, Lys, Ala, Orn, Ser(Ac), Sar, D-Arg and D-Lys;

R^3 is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc, Lys and Tyr;

R^4 is selected from the group consisting of Tyr, $Tyr(PO_3)_2$, Thr, Ser, Ala, homoSer and azaTyr;

R^5 is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

R^6 is His, Arg or 6-NH_2-Phe;

R^7 is Pro or Ala; and

R^8 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, excluding sequences including R^4 as a terminal Tyr group,

wherein the active agent is not AII; and

(b) instructions for using the amount effective of active agent to augment erythropoiesis.

16. The kit of claim 15 wherein the active agent is selected from the group consisting of angiotensinogen, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO: 32, SEQ ID NO:33, SEQ ID NO:

34; SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, and SEQ ID NO:39.

17. The kit of claim 15 wherein the concentration of active agent is between about 0.1 ng/ml and about 10.0 mg/ml.

18. A kit for augmenting erythropoiesis comprising an amount effective to augment erythropoiesis of an active agent comprising a sequence of the following general formula:

ASP-ARG-R1-R2-R3-R4-PRO-R5

wherein R1 is selected from the group consisting of Val, Pro, and Lys;

R2 is selected from the group consisting of Tyr, Tyr $(PO_3)_2$ and Ala;

R3 is selected from the group consisting of Ile, Val, Leu, norLeu and Ala;

R4 is selected from the group consisting of His and Arg; and

R5 is either Phe or is absent,

and wherein the active agent is not AII; and

(b) instructions for using the amount effective of active agent to augment erythropoiesis.

19. The kit of claim 18 wherein the active agent is selected from the group consisting of SEQ ID NO:4, SEQ ID NO:19, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:31, SEQ ID NO: 32, SEQ ID NO: 34; SEQ ID NO:38, and SEQ ID NO:39.

20. The kit of claim 18 wherein the active agent is selected from the group consisting of SEQ ID NO:4, SEQ ID NO. 31, SEQ ID NO 38, and SEQ ID NO. 39.

21. The kit of claim 18 wherein the concentration of active agent is between about 0.1 ng/ml and about 10.0 mg/ml.

22. An improved cell culture medium for promotion of erythropoiesis, wherein the improvement comprises addition to the cell culture medium an amount effective to promote erythropoiesis of at least one active agent comprising a sequence consisting of at least three contiguous amino acids of groups R^1-R^8 in the sequence of general formula I

$$R^1\text{-}R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7\text{-}R^8$$

in which R^1 and R^2 together form a group of formula

$$X\text{-}R^A\text{-}R^B\text{-},$$

wherein X is H or a one to three peptide group,

R^A is suitably selected from Asp, Glu, Asn, Acpc (1-aminocyclopentane carboxylic acid), Ala, Me^2Gly, Pro, Bet, Glu(NH_2), Gly, Asp(NH_2) and Suc,

R^B is suitably selected from Arg, Lys, Ala, Orn, Ser(Ac), Sar, D-Arg and D-Lys;

R^3 is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc, Lys and Tyr;

R^4 is selected from the group consisting of Tyr, Tyr(PO_3)$_2$, Thr, Ser, Ala, homoSer and azaTyr;

R^5 is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

R^6 is His, Arg or 6-NH_2-Phe;

R^7 is Pro or Ala; and

R^8 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, excluding sequences including R^4 as a terminal Tyr group,

and wherein the active agent is not AII.

23. The improved cell culture medium of claim 22 wherein the active agent is selected from the group consisting of angiotensinogen, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO: 32, SEQ ID NO:33, SEQ ID NO: 34; SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, and SEQ ID NO:39.

24. The improved cell culture medium of claim 22 wherein the concentration of active agent is between about 0.1 ng/ml and about 10.0 mg/ml.

25. An improved cell culture medium for promotion of erythropoiesis, wherein the improvement comprises addition to the cell culture medium an amount effective to promote erythropoiesis of at least one active agent comprising a sequence of the following general formula:

ASP-ARG-R1-R2-R3-R4-PRO-R5

wherein R1 is selected from the group consisting of Val, Pro, and Lys;

R2 is selected from the group consisting of Tyr, Tyr $(PO_3)_2$ and Ala;

R3 is selected from the group consisting of Ile, Val, Leu, norLeu and Ala;

R4 is selected from the group consisting of His and Arg; and

R5 is either Phe or is absent,

and wherein the active agent is not AII.

26. The improved cell culture medium of claim 25 wherein the active agent is selected from the group consisting of SEQ ID NO:4, SEQ ID NO:19, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:31, SEQ ID NO: 32, SEQ ID NO: 34; SEQ ID NO:38, and SEQ ID NO:39.

27. The improved cell culture medium of claim 25 wherein the active agent is selected from the group consisting of SEQ ID NO:4, SEQ ID NO. 31, SEQ ID NO 38, and SEQ ID NO. 39.

28. A novel peptide with erythropoiesis promoting activity, consisting of the sequence Asp-Arg-Lys-Tyr-Ile-His-Pro-Phe [SEQ ID NO:39].

29. A pharmaceutical composition comprising the peptide of claim 28 and a pharmaceutically acceptable carrier.

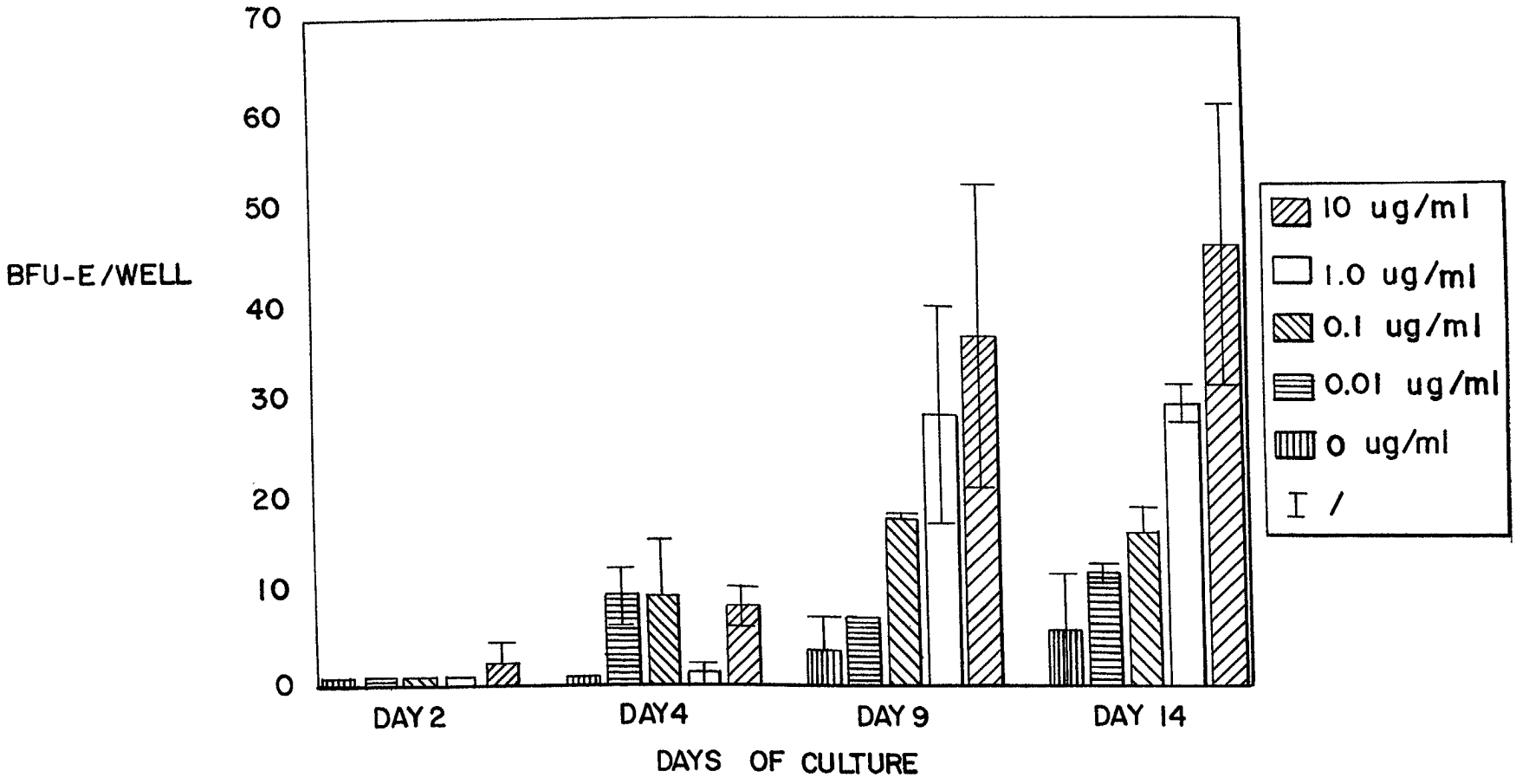
30. The pharmaceutical composition of claim 29 further comprising an amount of erythropoietin effective to stimulate erythropoiesis.

Abstract of the Disclosure

The present invention provides methods, compounds, pharmaceutical compositions, and kits for the augmentation of erythropoiesis by potentiating erythropoietin-induced differentiation with angiotensinogen, angiotensin I (AI), AI analogues, AI fragments and analogues thereof, angiotensin II analogues, AII fragments or analogues thereof or AII AT_2 type 2 receptor agonists as a therapeutic adjunct. The method is useful for the treatment of congenital or acquired aplastic or hypoplastic anemia associated with chronic renal failure, end-stage renal disease, renal transplantation, cancer, AIDS, chemotherapy, radiotherapy, bone marrow transplantation and chronic diseases.

FIG. 1

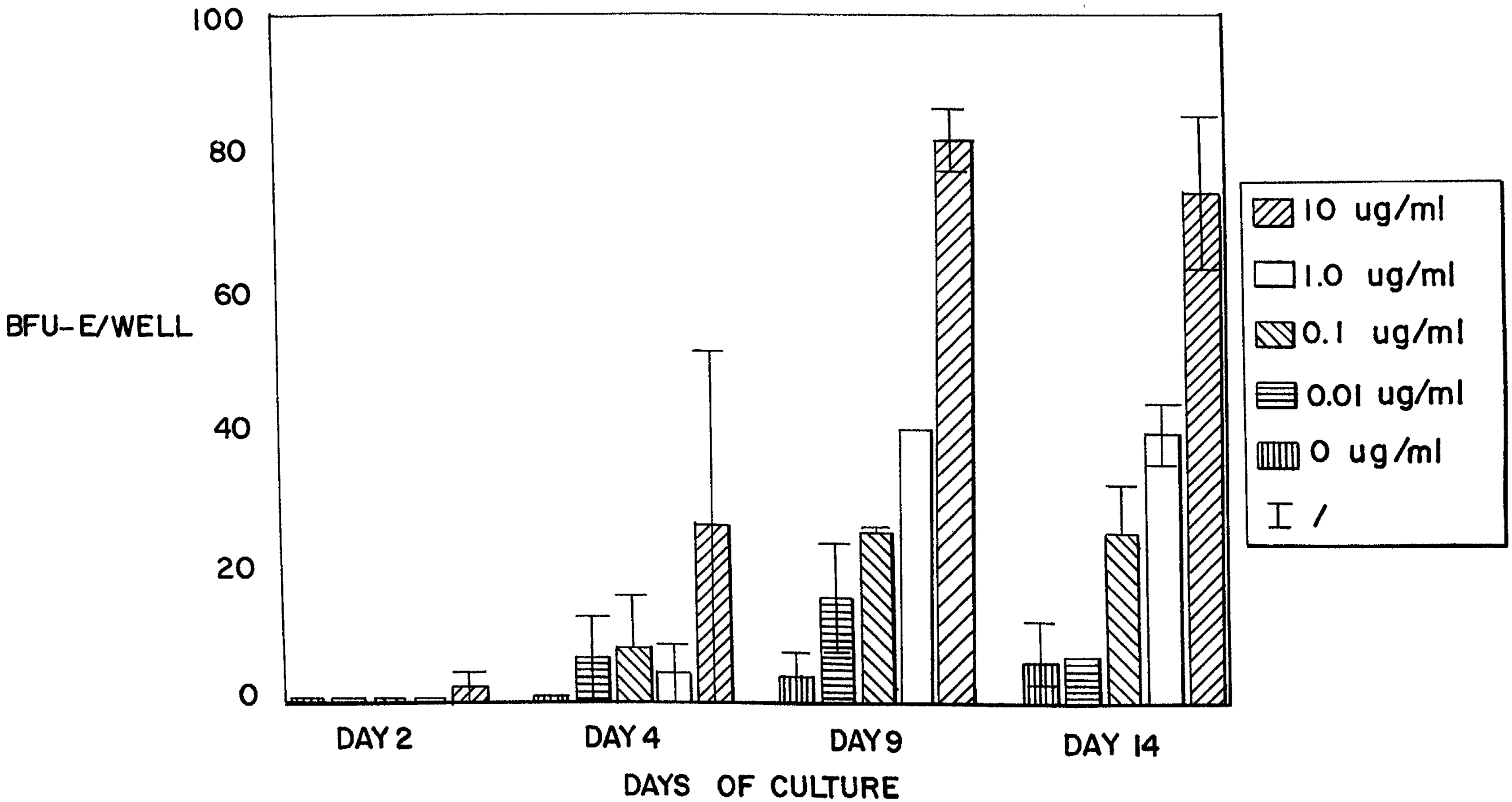
EFFECT OF IGD ON FORMATION
OF HUMAN BURST FORMING UNITS-ERYTHROID



CD 34+ CELLS, CORD BLOOD

FIG. 2

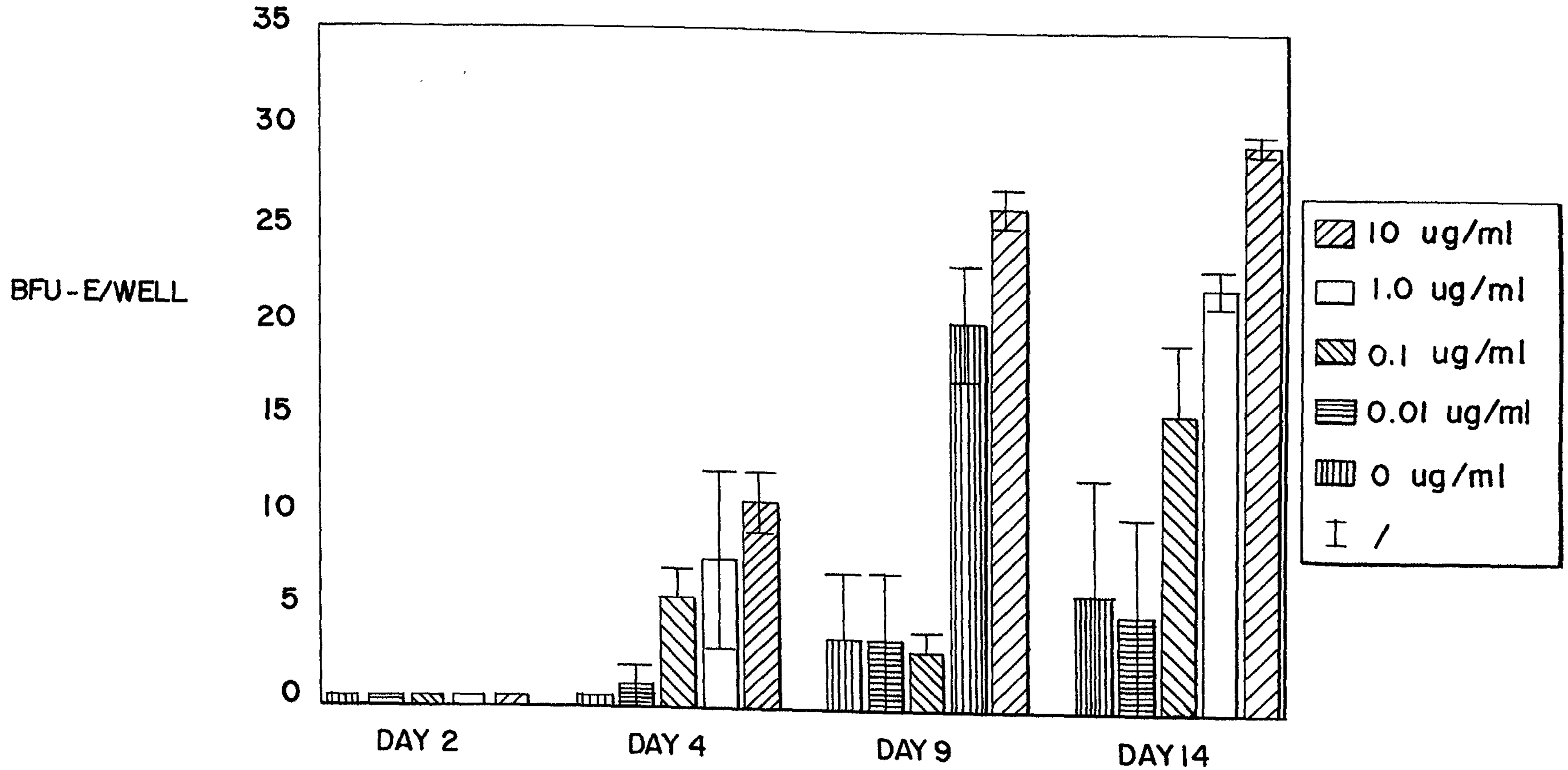
EFFECT OF 24B ON FORMATION
OF HUMAN BURST FORMING UNITS - ERYTHROID



CD 34+ CELLS, CORD BLOOD

FIG. 3

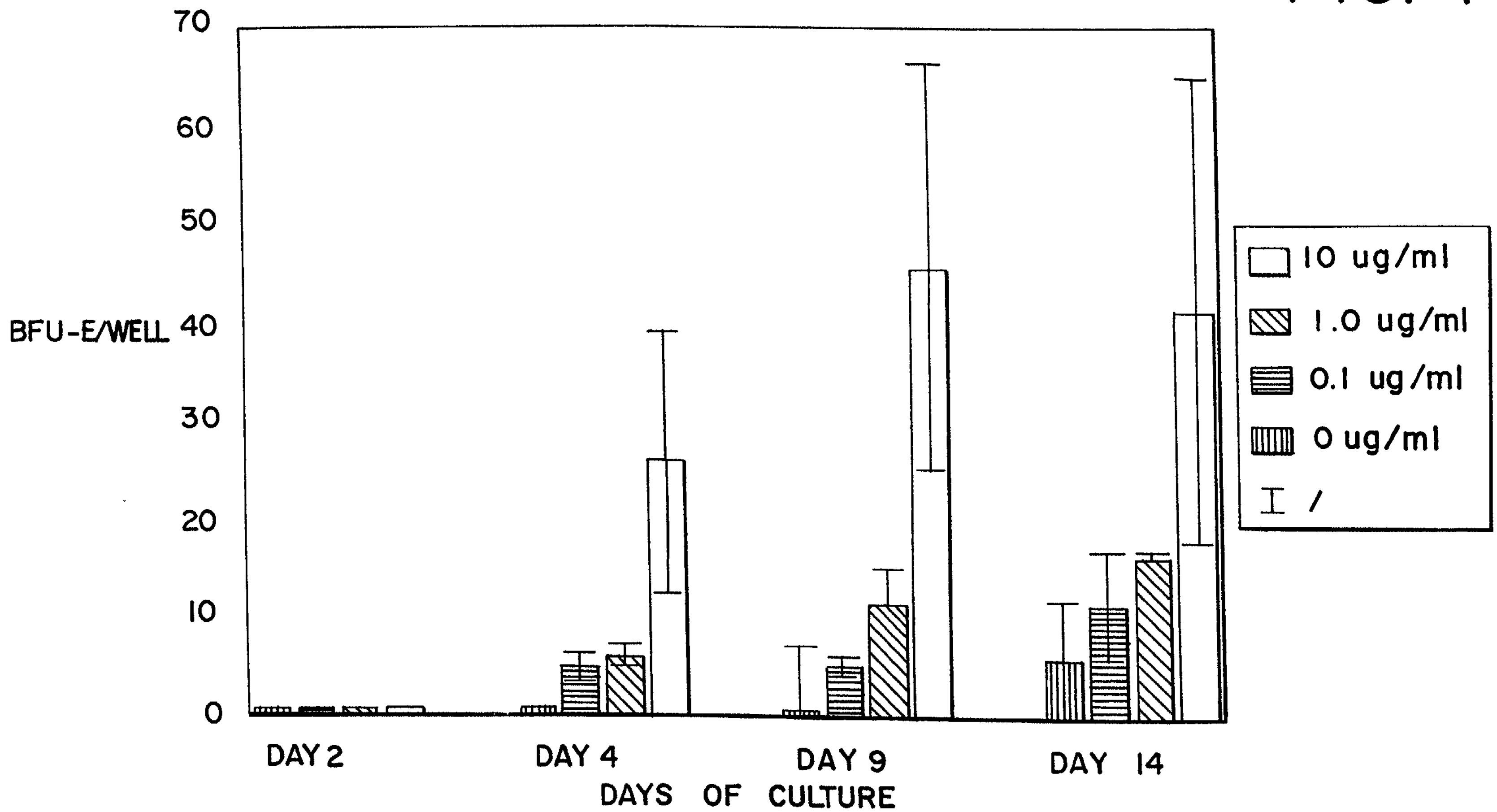
EFFECT OF 2GD ON FORMATION OF HUMAN BURST FORMING UNITS-ERYTHROID



CD 34+CELLS, CORD BLOOD

FIG. 4

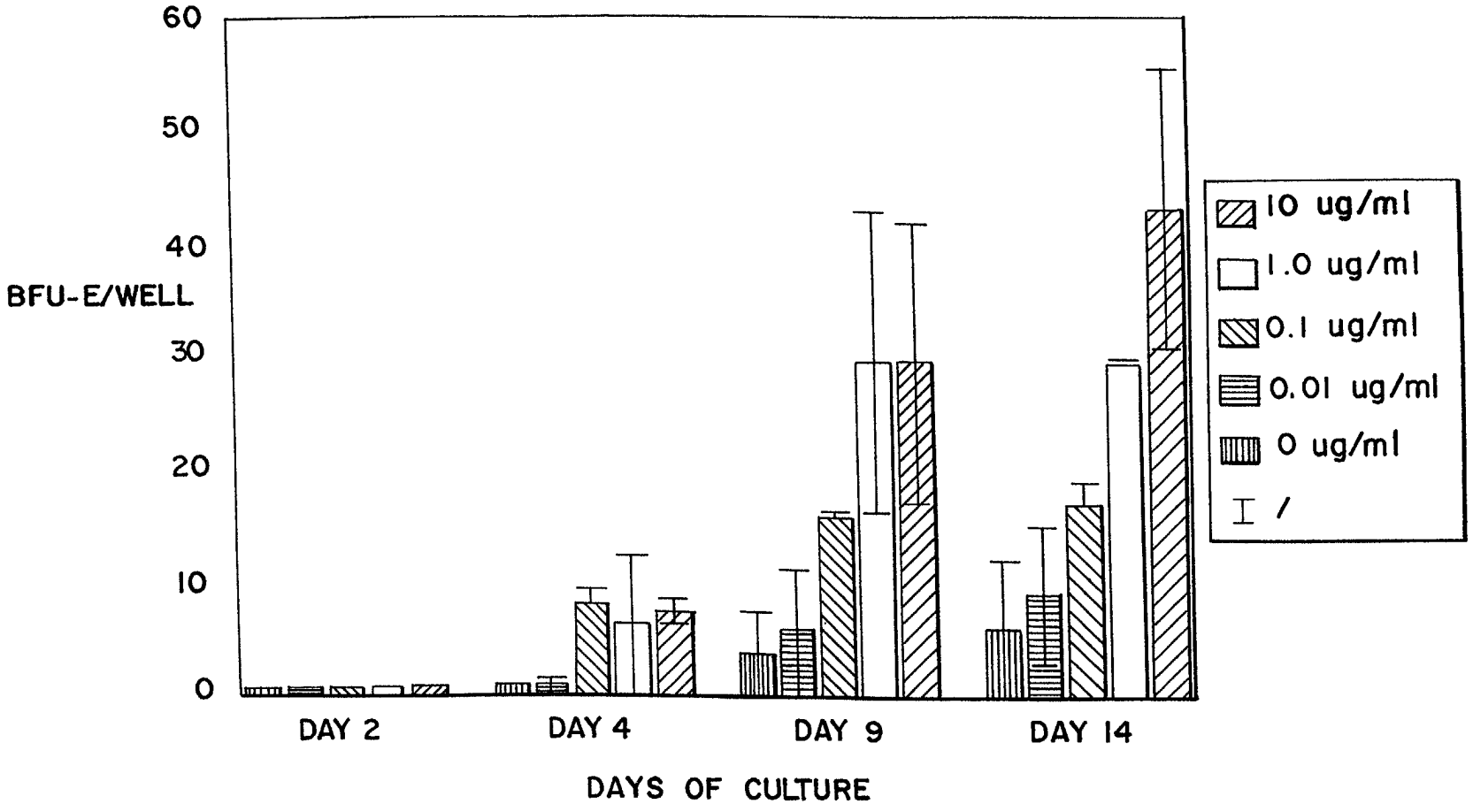
EFFECT OF 5GD ON FORMATION
OF HUMAN BURST FORMING UNITS-ERYTHROID



CD 34 + CELLS, CORD BLOOD

EFFECT OF ALLI-7 ON FORMATION
OF HUMAN BURST FORMING UNITS-ERYTHROID

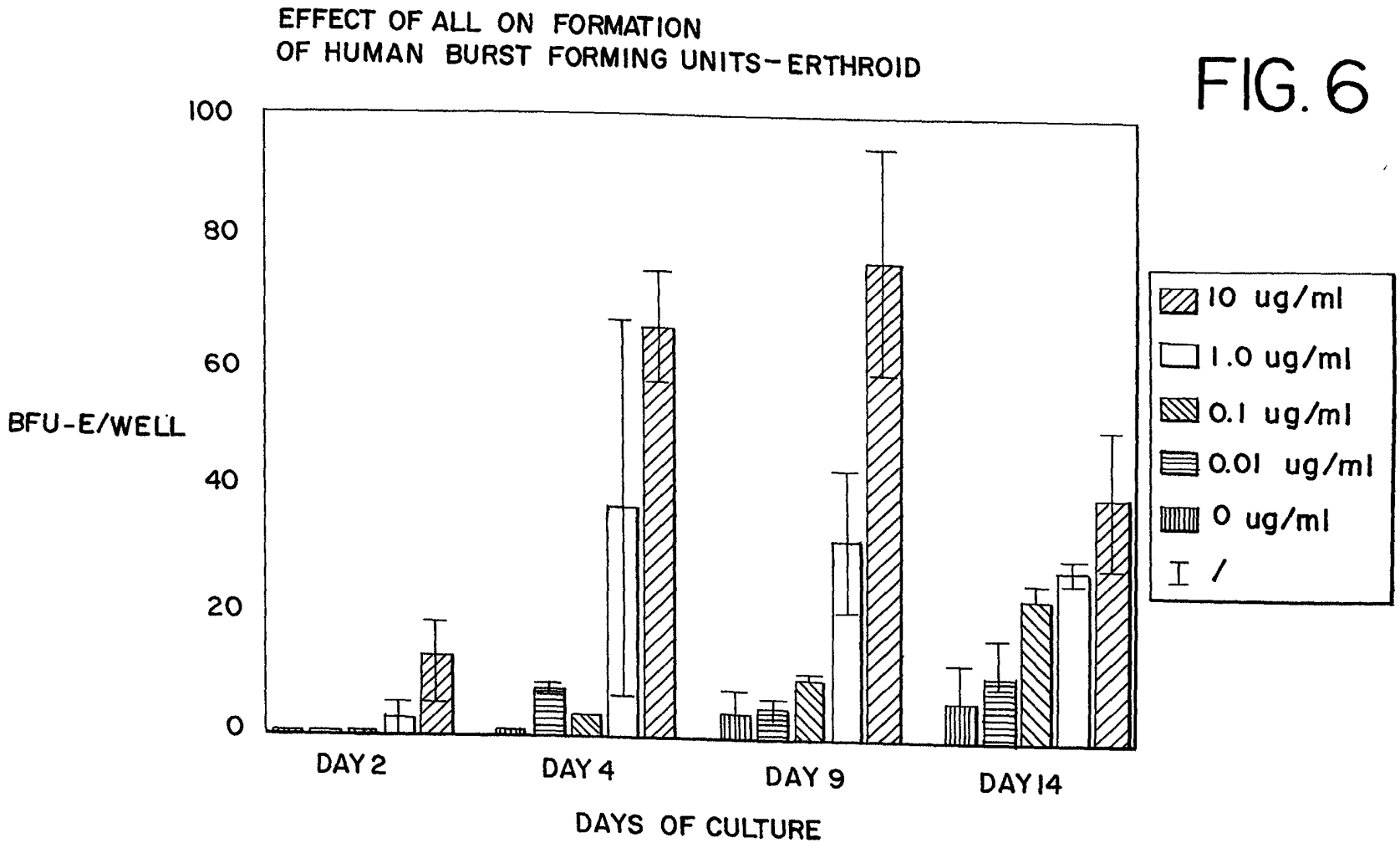
FIG. 5



CD 34+CELLS, CORD BLOOD

08868 "Stessel"

FIG. 6



CD 34+ CELLS, CORD BLOOD

Case No.: 98,009-B

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

METHOD OF PROMOTING ERYTHROPOIESIS

the specification of which is attached hereto unless the following space is checked:

☒ was filed on **February 8, 1999** as United States Application Serial Number **09/245,680**.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT international application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s):

	Number	Country	Day/Month/Year Filed
1.			
2.			

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below:

	Application Number	Filing Date
1.	**60/074,106**	**February 9, 1998**
2.	**60/111,535**	**December 9, 1998**

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or § 365(c) of any PCT international application designating the United States, listed below and, insofar as

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I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or § 365(c) of any PCT international application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R. § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

	Application Number	Filing Date	Status: patented, pending, abandoned
1.			
2.			

I hereby appoint the following attorneys and agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor (given name, family name): Kathleen E. Rodgers

Inventor's signature: Kathleen Rodgers Date: 1/28/99
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Citizenship: United States of America
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Full name of second or joint inventor (given name, family name): Gere DiZerega

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
(Case No. 98,009-B1)

In the Application of:	)	
	)	
Kathleen Rodgers, et al.	)	
	)	**Examiner: To be assigned**
Serial No.: To be assigned	)	
	)	**Group Art Unit: To be assigned**
Filing Date: Herewith	)	
	)	
For: Method of Promoting Erythropoiesis	)	

SUBMISSION OF SEQUENCE LISTING

Asst. Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

The Applicant herein submits a Sequence Listing in paper and computer readable forms.

The two forms are the same and neither contains new subject matter.

Respectfully submitted,

Date: September 8, 2000

David S. Harper
Registration No. 42,636

McDonnell Boehnen Hulbert & Berghoff
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Telephone: 312-913-0001
Facsimile: 312-913-0002

SEQUENCE LISTING

<110> Rodgers, Kathleen
diZerega, Gere

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<140> To be assigned
<141> 2000-09-08

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<170> PatentIn Ver. 2.0

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<210> 23
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<210> 24
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<210> 25
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<400> 25

Asp Arg Val Thr Ile His Pro Phe
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<210> 26
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<210> 27
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<210> 28
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<400> 28
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<210> 29
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<210> 30
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<210> 31
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<210> 32
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<400> 33
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1 5

<210> 35
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1 5

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Asp Arg Val Tyr Ile His Pro Phe His Leu

1 5 10

<210> 38
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Pro3-AII(1-7)

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1 5

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AII(1-7)

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Asp Arg Lys Tyr Ile His Pro
1 5